Effect of Simulated Hypergravity on Germination, Growth and Secondary Metabolites Production of *Eruca Sativa* Mill.

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Abstract

Eruca sativa Mill. (Arugula) belongs to the Brassicaceae family and is widely used in cooking in many countries. When used in therapeutic treatment, it has been shown to demonstrate antioxidant, anticarcinogenic, antimicrobial and antifungal activity. This activity has been attributed to its secondary metabolites, the glucosinolates and their hydrolysis products, isothiocyanates. These characteristics prompted an evaluation of the effect of simulated hypergravity on this species, since this condition has already demonstrated morphological and physiological changes in several other plant species, such as increased growth and modification of intracellular components. In this study, the condition of simulated hypergravity was accomplished through application of centrifugal acceleration to +7 Gz. The results demonstrated no significant difference in germination, growth and phenolic compound production between the arugula control and centrifugal groups. The composition and distribution of essential oil differed between the two groups, however, both oils presented antifungal activity for *Aspergillus niger*. The major component of the essential oil of the centrifugal group was 1-isothiocyanato butane. This was not found in the control group, suggesting that simulated hypergravity can be used to increase production of secondary metabolites of pharmaceutical interest.

Keywords: Simulated Hypergravity, Centrifuge, Secondary Metabolites, *Eruca sativa* Mill.

INTRODUCTION

Plants undergo numerous physical stimuli during their growth, which can modify their physiology and morphology (CENTILES-AUBAY et al., 2003). Among these stimuli are light, temperature, water and gravity (Wakabayashi et al., 2005). Although still little explored, gravity has an influence on plant development through gravitropism, which is a phenomenon observed in response to the direction of gravity acceleration (Toyota et al., 2007). Plant leaves are directed toward sunlight and their root system toward the ground as a result of this, enabling fixation of the plant and adequate absorption of nutrients and minerals (Hoson T, Wakabayashi, K et al., 2014).

Gravity, as well as other environmental stimuli, is perceived mainly in the plant cell walls (Hoson et al., 2005). This is the main part of the plant where chemical changes related to these stimuli occur (Wakabayashi et al., 2005). These cell wall alterations may also indicate a relationship with another type of phenomena, such as resistance to gravity.

Simulated hypergravity experiments have demonstrated mechanisms involved in this resistance to the force of gravity that are independent of gravitropism, which act as mechanoreceptor membranes and are essential in a plant's perception of the environment (HOSON et al., 2005).

The *Eruca sativa* Mill., popularly known as arugula, belongs to the Brassicaceae family and has been cultivated in the Mediterranean since ancient times (PASINI et al., 2012). Although its use is culturally associated with cuisine, arugula has also been the subject of study by the scientific community, due mainly to its secondary metabolites, the glucosinolates. These metabolites and their hydrolysis products, isothiocyanates and nitriles, have exhibited significant pharmacological power as potent antioxidants and anticarcinogenics (PAULINO et al., 2008). Moreover, these components are responsible for the aroma and flavor characteristic of this species (PASINI et al., 2012).

Studies involving the effects of simulated hypergravity on different plant species have presented an increase in both the number of germinated seeds and growth of plants (RUSSOMANO et al., 2007, SANTOS et al., 2012), in addition to providing cellular (TOYOTA et al, 2007) and metabolism (KOIZUMI et al., 2007; Allen et al., 2009; SOGA et al., 2007) alterations. In this context, this research aimed to evaluate the effect of simulated hypergravity on the germination and growth, phenolic compounds and essential oils, and antifungal activity of *Eruca sativa* Mill.

MATERIALS AND METHODS

Simulated hypergravity

Experiments involving the application of simulated hypergravity on arugula seeds were performed through use of a centrifuge, developed by the Aerospace Engineering Laboratory of the Microgravity Centre - FENG/PUCRS. The centrifuge consists of two main structures and an electromechanical motor system. The base structure is formed of carbon steel and houses the electromechanical motor system. This structure connects to another in which supports are located for 12 sample holders. The desired speed in revolutions per minute (rpm) is established by changing the power supply voltage and through measurement of the rotation frequency using an optical non-contact tachometer. A digital timer is used to control time and an on/off system. For this study, a rotation frequency of 137 rpm was used, resulting in a final acceleration of +7Gz at the point where the samples were located.

Plant Material

The choice of plant species for performance of the experiment was based on plant characteristics, such as germination time (4-10 days), and its market availability at low-cost. On this basis, *Eruca sativa* Mill. (Isla Pak brand) seeds were chosen as the plant model to be exposed to simulated hypergravity using the previously described centrifuge.

Each sample holder contained three pieces of 18cm x 6cm germination paper, dampened with distilled water and having 15 seeds located on each piece, resulting in a total of 45 seeds per sample holder. Seeds were kept hydrated by adding 80mL of distilled water per sample. A layer of plastic film was used to cover the containers to reduce water loss through evaporation, but having perforations to allow air exchange. A total of five experiments were performed.

Simulated hypergravity was applied intermittently using the centrifuge, i.e., 8h in simulated hypergravity and 16h at rest. This cycle was repeated for 4 consecutive days. Control group samples were prepared in the same manner and maintained at rest (1Gz) under the same light, temperature and humidity conditions.

The number of seeds germinated in both groups was visually assessed at the end of the experiment protocol. The size of each seedling (root + shoot) was measured in cm using a ruler.

Essential Oil Extraction

Essential oil extraction for the centrifuge and control groups was conducted through a hydrodistillation technique connected to a Clevenger apparatus. The plants were placed in a 250mL round-bottom flask, immersed in distilled water to fill half the flask, which was then heated. The water vapor produced by the heating process carried the volatile components, which were condensed and then collected (BORSATO et al., 2007). The extraction process occurred over a period of 3h30min. To remove the oil from the pipette Clevenger, it was washed with 10 ml of hexane (4 x 2.5mL). The solution (oil + water + n-hexane) was placed in a pear separation and the hexane phase removed and put in amber vials containing anhydrous sodium sulphate, in order to ensure the adsorption of residual water. The vial flasks were kept refrigerated to avoid loss of material by volatilization or degradation.

Plants obtained from all the experiments underwent the same extraction process described above.

2.4 Chromatographic analysis of essential oils

Analysis of the arugula essential oil was accomplished by grouping the plants obtained from experiments 3, 4 and 5, to have sufficient plant material for oil extraction.

2.4.2 Gas chromatography coupled to mass spectrometer detector (GC/MS)

Analysis of the arugula essential oil chemical composition was performed using a gas chromatography method coupled to a mass spectrometer detector, at the LOPE (Laboratório de Operações Unitárias - Unit Operations Laboratory) PUCRS. The analysis equipment used was a Hewlett Packard - Agilent GC/MS system model 7890A GC and mass detector model 5975C. The column used was HP-5MS (Hewlett Packard - Agilent, 5% phenyl methyl silox, 30m x 250µm internal diameter with film thickness of 0.25µm).

The oven temperature program started at 60°C, maintained for 4min and then increased by 3°C/min until reaching 240°C, remaining at that temperature for 15min. The carrier gas used was helium with a flow of 1ml/min, injector temperature of 250°C, and injection volume of 2µl in splitless mode. Use of the assay conditions was determined according to literature data and tests.

2.5 Histological cutting

The cotyledons of the control and centrifuge groups were separated and sectioned freehand with the help of cutting blades. These cuttings were then observed, analyzed and photographed by means of a Zeiss Axiostar model optical microscope with a magnitude of 400x, using an attached Coolpix camera.

2.6 Determination of total phenolic compounds

Quantification of the phenolic compounds was performed through the Folin-Ciocalteu method (SOUSA et al., 2007), with 99% gallic acid as the standard reference. A 0.5g rocket plant sample for each group was weighed separately and placed in an electric mill containing 10ml of 80% methanol. The samples were then centrifuged at 4000rpm for 10min at 15°C. Each sample of 100µL was transferred to test tubes, followed by the addition of 1.5ml of water and 100mL of Folin reagent. The tubes were shaken, and after two minutes of rest, 300µL of 0.2% Na_2CO_3 was added. The mixture was stored in a dark place for 30min. The absorbance was measured at 765nm using a Femto 700 Plus spectrophotometer. The results were expressed as gallic acid equivalents (mg/g).

2.7 Bioauthography

Bioautography, a method combining thin layer chromatography (TLC) with a bioassay *in situ,* was performed according to the Rahalison et al., (1991) method. It was used to evaluate the antifungal activity of the *Eruca sativa* Mill. essential oil.

The aqueous phase of the control and centrifuge groups were transferred separately to a separation funnel and submitted to partitioning with different solvents in increasing order of polarity: dichloromethane, ethyl ethyl and n-butanol (3 X 4ml each solvent extraction). The fractionated extracts were concentrated in a Büchi rotary evaporator under reduced pressure. The residue

from each fraction was subjected to TLC. The eluent used was selected through a Microcircular selection method of eluents (SIQUEIRA et al., 2003). Three different eluents were tested: butanol / acetic acid / distilled water (4: 1: 1); toluene / ethyl acetate (10: 3); and hexane / ethyl acetate (7: 3). The extracts were placed on a GF254 silica plate and each eluent was applied to the same site as the extracts. Ultraviolet light (UV 360nm) was used to evaluate the plates. The fractions obtained with increasingly polar solvents and essential oil underwent bioauthographic analysis.

To perform this analysis, GF254 silica plates cut in the shape and size of a Petry plate were used, and the arugula plant hexane, ethyl acetate, dichloromethane and n-butanol extracts from both the control and centrifuge groups were applied separately. Ketoconazole at a concentration of 5 mg/ml was applied as a positive control for the inhibition of fungal growth. Negative controls, which were pure solvents, hexane, ethyl acetate, dichloromethane and n-butanol, were also used.

The silica plates were subsequently introduced into Petry plates and covered with 10-15ml of Sabouraud agar culture medium, specific for the culture of fungi already inoculated with *Aspergillus niger* spores. The Petry plates were closed and kept in an incubator at 37°C for 72 hours to observe the fungi growing. The plates were placed upside down to prevent water droplets, secondary to condensation, from falling on the board. These plates were prepared in duplicate with the extracts of the control and centrifuge groups, totaling 4 plates.

3. DATA ANALYSIS

The experiment results were analyzed using Student's "t" test, with one-way analysis of variance (ANOVA), from the SPSS software, version 11.5.

4. RESULTS

4.1 Germination and growth

Analysis of the germinated seeds found no statistically significant difference ($p = 0.2$) between the centrifuge and control groups. Of the 2160 seeds used in the experiment, 181 in the centrifuge group and 206 in the control group failed to germinate. A total of 34 centrifuge and 28 control group seeds germinated but did not continue growing to generate a root or shoot. For those seeds that continued to grow, the shoots observed for both groups were similar, but slightly more root development was seen in the group that underwent simulated hypergravity. When considering the total plant growth by addition of the root and shoot portions, a tendency toward greater growth of seedlings in the centrifuge group was noted, as shown in Figure 1.

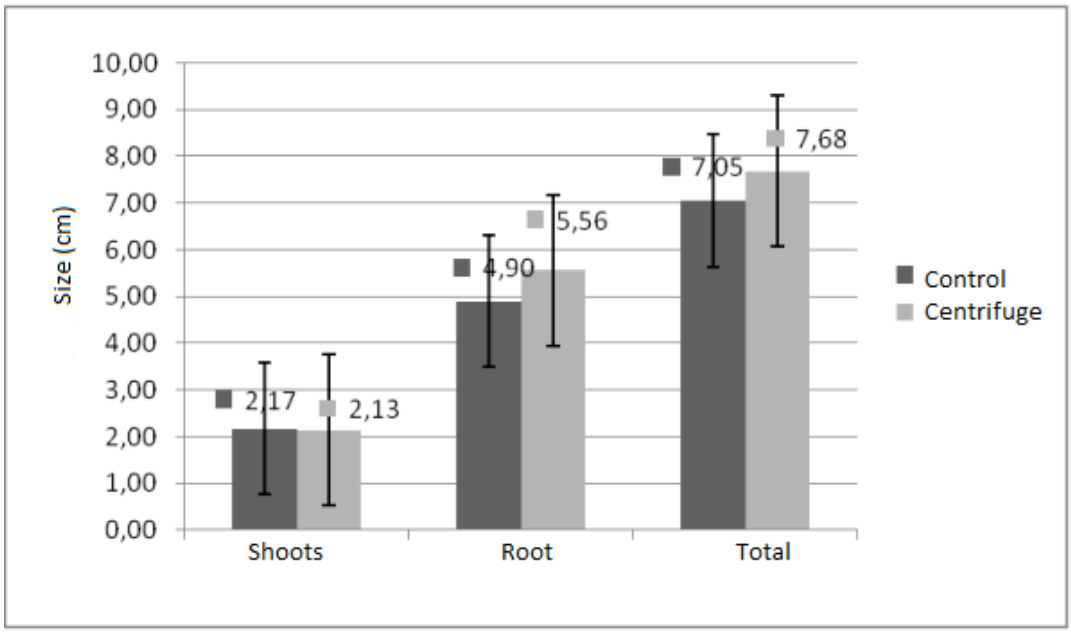

Figure 1 - Comparison of *Eruca sativa* Mill. growth between the control and centrifuge groups, including the shoot, root and total growth. Source: VIEIRA 2007.

4.2 HISTOLOGICAL CUTTING

Histological analyses of the cotyledon showed a difference in the distribution of essential oils, with the centrifuge group presenting a more peripheral distribution (Figure 2). Conversely, the essential oil of the control group was located in the middle of the cell (Figure 3), suggesting that simulated hypergravity influenced the distribution of the arugula essential oils, even after the simulated hypergravity ended.

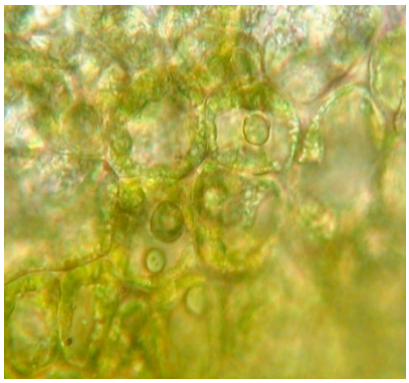

Figure 2 - Histological analysis of the control group cotyledon showing a centralized distribution of the essential oil (400x). Source: VIEIRA 2007.

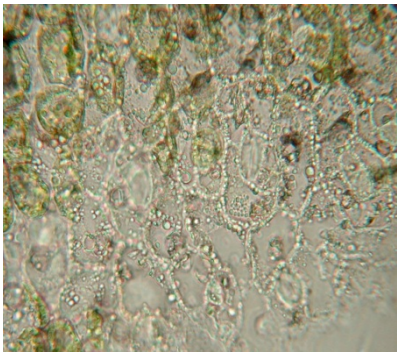

Figure 3 - Histological analysis of the control group cotyledon showing a more peripheral distribution of essential oils (400x). Source: VIEIRA 2007.

4.3 DETERMINATION OF TOTAL PHENOLIC COMPOUND CONTENT

The amount of phenolic compounds produced by the arugula did not differ when the two groups were compared ($p = 0.06$). It is believed this happened as the statistical analysis was performed during the seedling phase, before plants have achieved their adult stage.

4.4 COMPOSITION OF ESSENTIAL OILS

It was possible to conduct analysis of the essential oils after the accumulation of plant material from 3 subsequent experiments. The total mass obtained was 25.72g in the centrifuge group and 20.89g in the control group. Therefore, the centrifuge group produced 18% more mass material than the control group. Table 1 presents the major components of both groups, being α-pinene and limonene in the control group, and the derivative of isothiocyanate, 1-isothiocyanato-butane in the centrifuge group, corresponding to almost 50% of the total composition of essential oil in this group. Despite it being the major compound present in the group that underwent simulated hypergravity, it did not appear in the control group. This compound is known in the scientific literature for having an anticancer action. This result suggests that simulated hypergravity promotes changes in the production of secondary metabolites of plants without genetic manipulation.

Table 1- Analysis by GC/MS chromatography of the *Eruca sativa* Mill essential oil components of both the centrifuge and control groups

	Centrifuge Group		Control Group	
Components[a]	**IR**[b]	**Area%**[c]	**IR**	**Area%**
3-hydroxy-4-phenil-2-butanone	4.697	1.350	-	-
Trans sabinyl acetate	-	-	4.973	1.050
N-nonane	5.800	1.710	5.976	1.300
Alpha-pinene	6.924	2.210	6.944	6.240

3-methyl-cyclohexanone	7.686	1.250	7.682	1.040
1,2,4-trimethyl benzene	7.918	1.790	7.918	1.050
Mesitylene	9.163	1.810	9.163	2.460
N-decane	9.467	3.380	9.464	2.460
2-phenyl propanal	10.366	1.090	-	-
Hexadecane	10.431	1.750	-	-
Ortho cymene	-	-	10.487	2.290
Limonene	10.670	2.540	10.726	10.740
Methyl lactate	10.778	1.150	-	-
1,8 cineole	-	-	10.801	3.240
N-undecane	13.991	2.460	13.991	2.390
Isopulegol	-	-	16.096	1.340
Citronellal	16.544	3.620	16.354	0.350
Isoquinoline	18.829	1.350	-	-
1-isothiocyanante butane	29.870	49.530	-	-

a = compounds identified by comparison of their mass spectra and retention indices with the library Adams (2007). Compounds with more than 1% in area were considered.

b IR = retention ratio calculated for a range of alkanes.

c Area% = percent area of each peak to the total area of the chromatogram.

Source: SQUENA 2007

In the bioautography test, only hexane fractions of the essential oil of both groups presented an inhibition zone for the microorganism *Aspergillus niger*, reproducing the same result obtained with the positive control (ketoconazole). The hexane solvent applied as negative control, however, showed no inhibition zone, confirming that the inhibition of microorganism growth was due to the essential oil *Eruca sativa* Mill. Bioauthographic analysis of the essential oil components of both the control and centrifuge groups after the TLC method of separation was conducted, employing acetic acid/butanol/distilled water as the eluent system, showed the centrifuge group essential oil as presenting two zones of inhibition. The first had an Rf value of 0.7, and the second of 0.9. The control plate treatment showed only a single halo with an Rf value of 0.9. These results suggest that the essential oil of arugula from the centrifuge group has two compounds with antifungal activity against *Aspergillus niger*. These components, however, have yet to be identified.

5. DISCUSSION

Many studies have shown that gravitational force can influence plants in various ways, such as increasing the amount of xyloglucan in plant cell walls (SOGA et al., 2007) and thickening inflorescence stems in *Brassica rapa* (Allen et al., 2009). Experiments performed by Russomano et al. (2007) found increased germination of arugula seeds and significantly more growth of the seedlings during hypergravity simulation than in the control group. It was suggested that the auxin was responsible for this cell growth. These findings, however, were not confirmed in our research. A comparison of the two experiments highlighted the only difference between them, being the substrate used by Russomano et al, which was soil. Germination paper was used in the present study. It is well known that seedlings use nutrients from the seeds to grow at the beginning of the plant cycle. Nutrients from the surrounding environment are only used later in the growth process. Therefore, it is believed that the noted differences occurred as the plants were already at a stage where environmental nutrients were needed when they were analysed, and no nutritional supplements were available, such as the soil used in the Russomano study (2007).

The secondary metabolites of arugula, the glucosinolates, are fairly widely reported in several studies (PAULINO et al., 2008; FIMOGNARI et al., 2004; BLAZEVIC et al., 2010; KHOOBCHANDANI et al., 2010). Its production in the plant can be influenced by the storage and amount of ascorbic acid present in the cells. These metabolites originate from a group of amino acids by a process that can be divided into three phases. The first occurs where the lengthening chain of amino acids exerts significant influence on the insect resistance function and anticancer activity of glucosinolates (PAULINO et al., 2008). In the second phase, conversion of the amino acid moiety to the glucosinolate structure occurs, and also in the third, which is characterized by oxidative modifications in the side chains. These compounds are stored in the vacuoles of the plant cells and are exposed to the action of myrosinase enzymes when they suffer any cell damage, which in turn are responsible for the hydrolysis of these compounds, resulting in isothiocyanates (FALK et al., 2004).

Isothiocyanate has the potential to prevent cancer due to its antiproliferative capacity and is also an apoptosis promoter (FIMOGNARI et al., 2004). Studies involving *Eruca sativa* Mill. using cyclohexane/ethyl acetate extract have also been tested in HepG2, a metabolically competent liver cancer cell line. The results suggest that the use of arugula extract produces a reduction of up to 5 times in the induced genotoxicity, when compared to cell cultures untreated with the same plant extract (LAMY et al., 2008). Furthermore, according to Azarenko et al., (2014) butane-1 isothiocyanate inhibits proliferation of MCF7 breast cancer cells (IC50 = 28µM) in parallel with cell cycle arrest at mitosis (IC50 = 13µM) and apoptosis, due to a mechanism that is consistent with the impairment of microtubule dynamics. Antimicrobial activity has also been reported, in addition to the anticarcinogenic activity. Research performed by Khoobchandani et al., 2010, revealed that *Eruca sativa* Mill. extract had antimicrobial activity against gram-positive bacteria, including *B. subtilis* and *S. aureus,* and gram-negative bacteria, such as *E. coli, S. flexneri*, and *P.*

aeruginosa. According to Land et al., (1992), the Brassicaceae family has been shown to present antifungal activity. Additionally, according to Shoaib et al., (2014) arugula oil can be used for anti-dandruff control. Studies have shown that glucosinolate compounds have antifungal activity (SOLEDADE M., C. STONES, HOSSAIN, S., 2011), as well as their derivatives, the isothiocyanates (MARI et al., 2002). A large quantity of the isothiocyanate compound was found only in the simulated hypergravity group in this study. In addition, in the hexane fractions of the TLC essential oil, a compound was found with an Rf value of 0.7, which presented an inhibition zone against *Aspergillus niger* in the chromatographic plate. This was not seen in the control group, suggesting that this compound could be the butane-1 isothiocyanate.

6. CONCLUSION

This research has demonstrated that the growth of seedlings can be dependent on the stage at which they are analyzed, however, it becomes independent when the substrate used is soil. It also suggests that the quantification of phenolic compounds should be conducted in adult plants, in order to confirm whether the use of simulated hypergravity increases or not the production of such compounds. It was possible to identify that the use of simulated hypergravity produced an 18% increase of plant mass, but more detailed analysis of the plant is required to better identify the significance of this finding, especially in relation to its nutritional components. The alteration in essential oil distribution in the histological sections of cotyledons is possibly due to the action of the centrifugal movement. However, it is not known if this modification is a result of some disruption of cell structure. It was proved that the essential oil of *Eruca sativa* Mill. has antifungal activity. Additionally, the results of this experiment suggest that simulated hypergravity stimulates the plant to produce secondary metabolites that are of therapeutic interest, such as anticancer compounds, without the need to apply genetic manipulation.

References

Azarenko, O; Jordan, M.A.; Wilson, L. Erucin, the Major Isothiocyanate in Arugula (Eruca sativa), Inhibits Proliferation of MCF7 Tumor Cells by Suppressing Microtubule Dynamics. **PLOS.ORG**, 2014.

Shoaib, A.; SAEED , G,; AHMAD, S, Antimicrobial activity and chemical analisys of some edible oils (clove, kalonji and tamarine). **African Journal of Biotechnology**, v.13(46): 4347-4354, 2014